

## CALCIUM PHOSPHATE GRANULES IN THE REPRODUCTIVE SYSTEM OF *OXYCHILUS ATLANTICUS* (GASTROPODA: PULMONATA)

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### ABSTRACT

The presence of connective interstitial cells in the wall of several organs of the distal reproductive system in *Oxychilus atlanticus* is described by means of scanning electron microscopy (SEM). Connective interstitial cells extend from the distal part of the vas deferens to the penis, being the dominant cell type of large portions of the epiphallus as well as in the whole penial caecum (=flagellum). These cells are highly mineralized and are characterized by the presence of large amounts of solid spherules (up to 4 µm in diameter) in their cytoplasm. A semi-quantitative evaluation by X-ray microanalysis demonstrates that calcium and phosphorus are the main elements of the spherules. Their function is unknown, but they could aid in copulation or they could assist the survival of the transferred spermatozoa.

### INTRODUCTION

*Oxychilus (Drouetia) atlanticus* (Morelet and Drouët, 1857) is a stylommatophoran snail of the Family Zonitidae, endemic to São Miguel Island, Azores. Several systematic studies have been undertaken on this species (Martins, 1991; Brito, 1992), mainly due to the great inter-specific variability. Presently, we are studying the structure and function of the reproductive system (Rodrigues, Gómez, Cunha & Martins, 1998; Rodrigues & Gómez, 1999) in order to advance in the knowledge of the reproductive biology of this species, a potential predator of *Lymnaea truncatula* (Müller), the intermediate host of the trematode *Fasciola hepatica* (L.) (Cunha, 1991). During the course of these investigations we have observed the presence of connective interstitial cells in the distal ducts of the reproductive system of *O. atlanticus*. These cells are characterized by the accumulation of great amounts of spheroid granules in their cytoplasm.

Characterization of accumulating cellular types in molluscs, as well as their physiological role, was first described by Fournié & Chetail (1982). According to these authors there are four accumulating calcium cellular types in molluscs: free connective cells, secretory subtegumentary cells, digestive gland calcium cells, and amebocytes.

Various forms of connective interstitial cells have been histologically described in the wall of the male reproductive system distal ducts in several stylommatophorans. Lusi (1961) and Smith (1965) working on *Arion rufus* (L.) and *Arion ater* (L.), respectively, describe them as vacuolated cells. Also in several helicids such as *Helix pomatia* Linnaeus and *Theba pisana* (Müller) interstitial round cells, which stain light with general histochemical methods have been identified in large amounts in the connective wall of the epiphallus (Noyce, 1973). They have also been recognized in the epiphallus of *Arion subfuscus* (Draparnaud) (Zubiaga, 1986) and *Arion hortensis* Férussac (Walls, unpublished in Runham, 1988). Histochemically, these vacuolated cells have been shown to contain non-sulphated acidic polysaccharides (Smith, 1965; Zubiaga, 1986), but no positive reaction has been obtained for calcium demonstration. Most of these interstitial cells have been described as secretory cells. Only for *Arion hortensis* they have been described as connective tissue cells containing large spherules resembling calcium granules (Runham, 1988) resembling those we have found in *Oxychilus atlanticus*. The presence of similar granules has also been indicated in several reproductive organs of *Arion rufus* (Lusi, 1961) such as the bursa copulatrix, the hermaphrodite duct, the vas deferens, the oviduct, and the atrium, and described as small amounts of large spherules, measuring up to 8 µm in diameter.

As far as we know the chemical composition of such granules has never been investigated. Scanning electron microscope energy-dispersive X-ray microanalysis has been undertaken in the present work to determine the main elements that constitute the composition of these structures in *O. atlanticus* in order to know their nature and to compare with other inorganic deposits subsequently found in other styломatophorans.

#### MATERIALS AND METHODS

Adult specimens of *O. atlanticus* were captured in the field in July, at the beginning of the mating season of this species. Copulating pairs, as well as non-copulating specimens were collected and immediately frozen by immersion in liquid nitrogen. Alternatively, specimens were kept alive in the laboratory until processing. In both cases, the soft parts were withdrawn from the shell and dissected under a stereomicroscope. Isolated reproductive distal organs, vas deferens, epiphallus, and penis with penial caecum (=flagellum of several authors working with zonitids) were dehydrated with ethanol (70%, 96%, 100%). In copulating specimens the whole copulatory organs, from the atrium to the bursa copulatrix, were removed and treated together. Fixatives or buffers were not used for the preparation of this material in order to avoid the potential contamination of tissues with exogenous chemical elements. To perform scanning electron microscopy (SEM) observations, the isolated organs were immersed once more in liquid nitrogen and a transverse cryofracture was made. Afterwards, these organs were returned to 100% ethanol. They were then treated in a critical point dryer (Balzers, CPD020), coated with gold in a Bal-tec SCD 004 Sputter coater, and observed in a JEOL SEM 6400. The X-ray microanalyses were performed with a energy-dispersive spectrometer (EDX) associated with the SEM microscope. The SEM was operated at 20 kV. Granules or spermatophore tunicle were probed centrally with the beam operating as a stationary spot. X-ray spectra were obtained with a Link microanalyser. The acquisition time was the same for all the analysis (100 seconds real time), thus allowing a direct comparison of the amount of metals between the different spectra.

#### RESULTS

The distal portion of the male genital system of *O. atlanticus* consists of vas deferens, epiphallus, and penis, with a penial caecum originating at the subterminal insertion of the epiphallus on the penis. In the female distal part there is a bursa copulatrix with its duct, a free oviduct, and the vagina with a perivaginal gland. A short common atrium opens on the body wall. During

the copulatory process there is both a progressive eversion of penis and intromission of this organ into the female ducts until the everted penial caecum reaches the bursa copulatrix lumen (Rodrigues & Gómez, 1999).

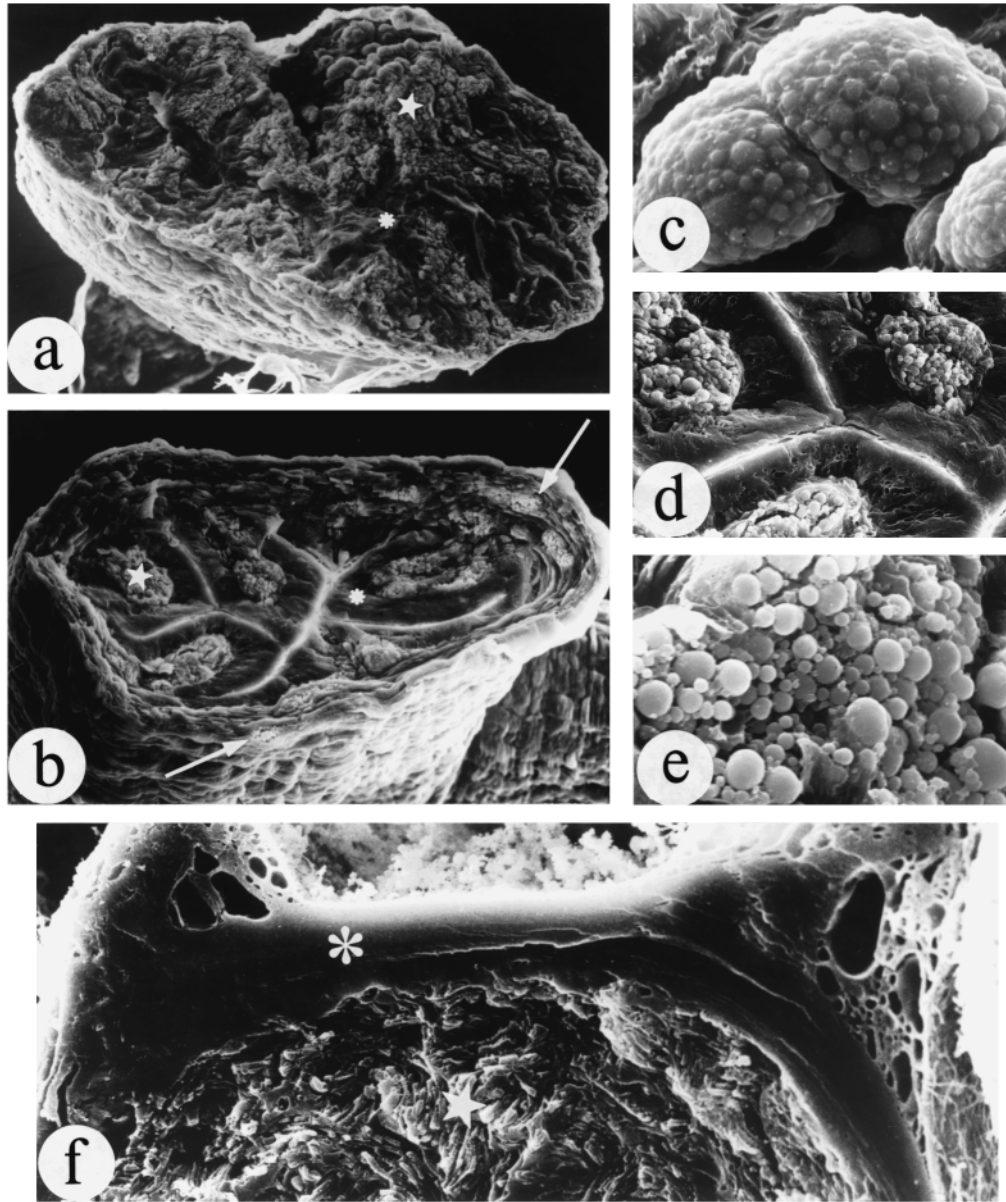
The penis is highly muscular with the wall thickening towards the atrium. About a quarter way from the atrium there is a conspicuous sheath of muscular-connective tissue which distally fuses with the penis wall. At the proximal end of the penis is a penial caecum (Fig. 1a); this is folded, and the wall slender and less muscular than the rest of penis. The columnar epithelium is surrounded by connective tissue with muscular fibres on it. A thin muscular layer, thicker in the distal penis, is external to this. The vas deferens is a long slender duct with three or four longitudinal folds. It is replaced distally by the epiphallus, a slightly enlarged duct with five or six longitudinal folds (Fig. 1b).

There are many granular cells in the connective wall of these organs. They have their cytoplasm full of granules, spheroidal in shape (Figs 1c,d,e). They vary in size, but the majority of them measure from 0.5  $\mu\text{m}$  to 2.5  $\mu\text{m}$  in diameter; the largest can measure up to 4  $\mu\text{m}$  in diameter. Transverse sections of the penial caecum show that this cell type represents almost all the connective element of its wall (Fig. 1a). It can also be the main cell type in the connective portion of the epiphallic folds (Figs. 1b,d). They are also present, but more scattered, in the distal portion of the vas deferens and in the proximal penis, beyond the penial caecum. These granular cells can also be observed in the thin outer muscular layer that surrounds all these male distal organs (Fig. 1b) as well as in the penial sheath. Several morula-like cell bodies of this cell type can be seen quite distinctly protruding on the external wall of the ducts (Fig. 2a,b), unless they are stretched; the roundness of the granules they contain is noticeable under the cell membrane (Figs. 1c, 2b). The X-ray microanalysis demonstrates that the main elements of these spherules are calcium and phosphorus (Figs. 1A + 3B). No other elements were detected. Elements with atomic number below that of sodium, such as carbon, hydrogen, nitrogen, and oxygen are not detected by the used techniques.

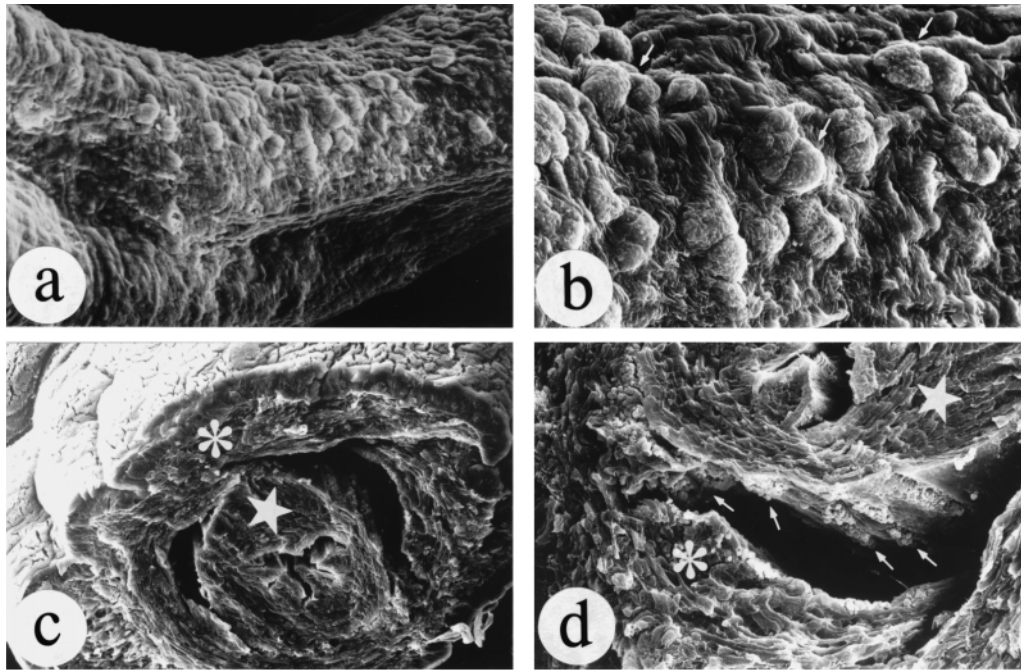
A transverse section made across the everted penis in copulating specimens shows the epiphallus being enclosed by the penis (Figs. 2c,d). The wall of the latter is turned over with the dense microvilli of its epithelium placed on the external surface (Fig. 2c) while the outer thin muscular layer of penis and epiphallus are face

to face. Several granular cells of both outer muscular layers are ripped and the spherules they contain released into the quite narrow space that exists between penis and epiphallus (Fig. 2d).

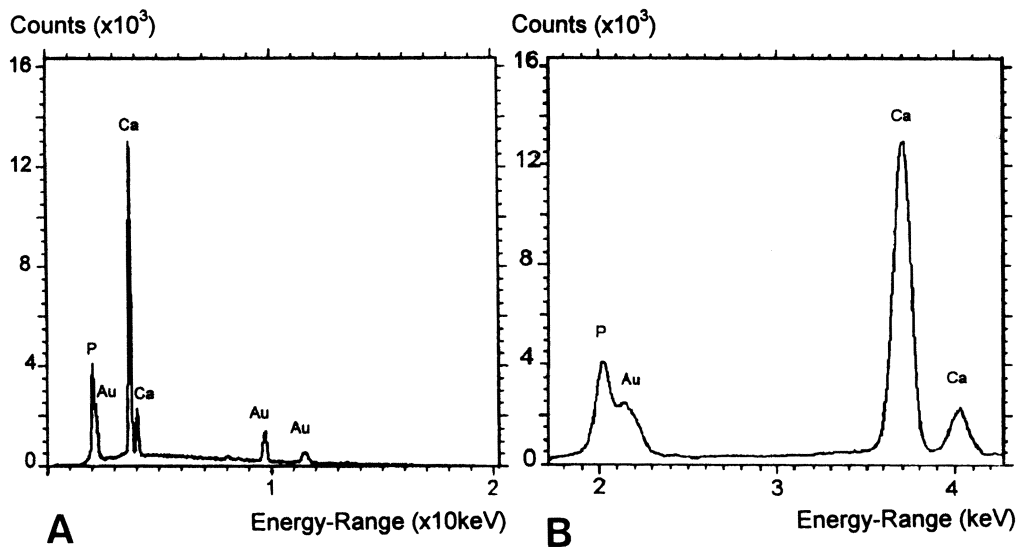
Frequently, it is possible to find a spermato-  
phore in specimens killed during copula. It can be found inside the proximal portion of the epiphallus, before having been transferred, or in the bursa copulatrix-pedunculus after sper-



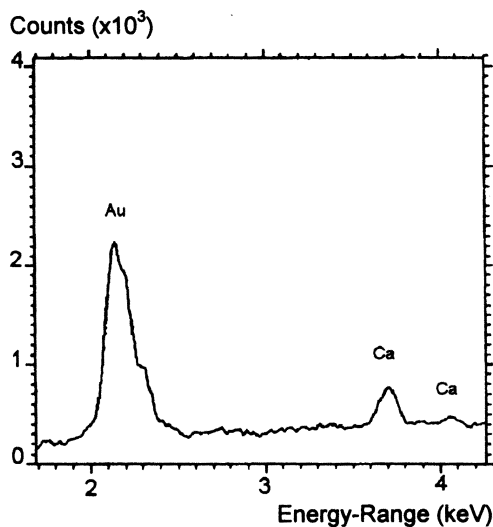
**Figure 1.** Scanning electron micrographs of distal male reproductive organs: **a.** Transversal view of penial caecum ( $\times 180$ ). The connective elements (star) are present between the epithelium (asterisk) and the outer envelope; **b.** Transversal view of epiphallus ( $\times 300$ ). There can be seen several folds with their epithelium (asterisk) and the connective granular cells (star). Arrows indicate the presence of several granular cells in the outer muscular layer; **c.** Detail of the morula-like cell bodies of granular cells in the penial caecum ( $\times 1800$ ); **d.** Detail of the internal folds of the epiphallus ( $\times 650$ ); **e.** Cytoplasmic spherules of the connective granular cells ( $\times 2200$ ).



**Figure 2.** Scanning electron micrographs of distal male reproductive organs: **a.** External view of the epiphallus ( $\times 170$ ); **b.** Detail of the wall of the epiphallus showing several morula-like cell bodies in the periphery ( $\times 400$ ); **c.** Transversal section of male ducts of a specimen killed during copula ( $\times 170$ ). The everted penis (asterisk) surrounds the epiphallus (star); **d.** Detail of the space that exists between penis (asterisk) and the epiphallus (star) in copulating specimens ( $\times 400$ ). Arrows indicate the presence of free spherules on the surface of both organs.



**Figure 3A,B.** X-ray microanalysis spectra of the spherules of the connective granular cells. **3A.** Full energy range. **3B.** Detail of the calcium and phosphorus peaks.



**Figure 4.** X-ray microanalysis spectrum of the spermatophore tunicle.

matophore transfer is completed. A section of the spermatophore of *O. atlanticus* shows the sperm core enclosed by a peripheral tunicle (Fig. 1f) as is usual for stylommatophorans. The X-ray microanalysis of both core and tunicle shows that only small amounts of calcium and no phosphorus are present in the spermatophore (Fig. 4).

#### DISCUSSION

Cells with solid spheroidal granules are found in a variety of tissues throughout the animal kingdom (Brown, 1982; Taylor & Simkiss, 1984). Large numbers of them are calcium containing granules that are to be found in specific cell types in the molluscs digestive gland or kidney (Taylor, 1995; Gibbs, Nott, Nicolaidou & Benianno, 1998). But there are some other cells containing granules in several tissues such as the connective tissue calcium cells, pore cells, and muscle (Fournié & Chetail, 1982; Simkiss & Mason, 1983). The role and function of these deposits are not well understood but they are considered to be a source of ions which can be mobilized when large amounts are needed, as in the shell synthesis and repair processes (Watabe et al., 1976) or during egg shell calcification (Tompa & Wilbur, 1977). Their role in metal detoxification has also been tested by several authors (Marigómez, Soto & Cajaville, 1995).

With respect to the genital system, there are several species which are thought to elaborate calcium reserves in the organs involved in the copula process. Nevertheless, the nature of their calcium has never been investigated but only suspected on the basis of their whitish colour. This is the case of the 'chalky gland' or the 'lime gland' of the Urocyclidae (Van Mol, 1970). Rigby (1963) says that the appearance of the five to six white longitudinal streaks which can be seen in the epiphallus of *Oxychilus cellarius* may be due to the accumulation of calcium salts in the connective tissue of the ridges.

Furthermore, several authors have indicated the presence of extracellular crystal-like structures placed on the surface of the penial system epithelium of various stylommatophores, sometimes embedded in a cuticular membrane. They have been reported in the Pupilloidea (Steenberg, 1925), in the Endodontoidea (Atkinson & Atkinson, 1987), or in the Helicoidea (Runham, 1988). Atkinson and Atkinson (1987) working with *Anguispira alternata* (Say) and *A. kochi* (Pfeiffer) demonstrated by EDX microanalysis the presence of large amounts of sulphur together with moderate concentrations of calcium, potassium, and phosphorus in the penial crystals of both species. Runham (1988) also comments on the presence of calcium-containing crystals in the lumen of the epiphallus of *Helix aspersa* and *Cepaea nemoralis* (L.). In *Gymnarion* (Urocyclidae) there is a thin capsule covering the top of penis (pers. observat.) that Binder (1976) refers to be produced by the lime gland of the epiphallus. Perhaps, the origin of the calcareous envelope that covers the penial papilla of the helioid *Cochlicella* (Schileyko & Menkhorst, 1997) could be related to these crystal precipitations.

The results obtained by the X-ray microanalysis in the present work proves that the granules of the male distal ducts of the reproductive system of *Oxychilus atlanticus* are composed mainly of calcium and phosphorus, probably in the form of calcium pyrophosphate ( $\text{Ca}_2\text{P}_2\text{O}_7$ ) as this is common in calcium phosphate deposits in a large number of invertebrates, including molluscs (Simkiss & Taylor, 1994). The intracellular deposits of calcium pyrophosphate are generally known to occur in large numbers in the pyramidal shaped 'calcium cells' in the molluscan digestive gland (Simkiss & Taylor, 1994; Marigomez et al., 1995; Gibbs et al., 1998), generally in the form of calcium magnesium diphosphate. Nevertheless, magnesium is virtually absent in the granules of the male distal ducts of *O. atlanticus*. Granules

associated with carbonate and phosphate show a spherical shape with a diameter ranging from 0.2 to 3  $\mu\text{m}$  (Taylor, 1995) which is in accordance with the morphology of the granules we have found in *O. atlanticus*.

A question that remains obscure is the physiological role of these spherules present in the reproductive ducts. On the basis of their calcium phosphate nature they could have a role in the acid-base control of the medium that surrounds the copulatory organs during copulation. It is widely known that carbonate and phosphate are the most common buffer systems in biology. These calcium deposits could also restore the intracellular calcium depletion caused by the prolonged stimulus of genital ducts during copula, which may take several hours in terrestrial gastropods (Tompa, 1984). In *O. atlanticus* it may last at least 24 hours (Rodrigues & Gómez, 1999). It has been documented (Randriamampita & Tsien, 1993) that intracellular calcium signals that last more than a few minutes after the onset of stimulation depend critically on influx of extracellular calcium. An alternative view is that the granules act as an energy store, regulating and releasing phosphate for functions involving ATP.

Another possible function is to contribute to synthesis of the spermatophore. It is known that the epiphallus (and flagellum when present) is the organ responsible for the formation of spermatophores, but only the peripheral tunicle is synthesized by the epiphallus (Rigby, 1963; Tompa, 1984; Runham, 1988). Koene and Chase (1998a) have found that only small amounts of calcium (mean values of 0.018 mg) are present in the spermatophore of *Helix aspersa*. This is in accordance with our findings that show a small calcium peak in the spermatophore of *O. atlanticus*. This low calcium content could probably be associated with the hardening of the spermatophore, as it has been previously suggested by several authors (Rigby, 1963; Runham, 1988). However, this can not explain the presence of inorganic deposits of calcium in the penis and penial caecum, because the formation of the spermatophore in *O. atlanticus* is restricted to the proximal half of the epiphallus (Rodrigues & Gómez, 1999).

During the copulation of *O. atlanticus* the penial caecum reaches the bursa copulatrix of the partner before spermatophore transference. Penial caecum is then everted, having its epithelium in close contact with the bursa copulatrix epithelium while the anterior end of the spermatophore is released directly into the bursa lumen (Rodrigues & Gómez, 1999). On the

basis of this behaviour, we think that the secretions of the penial caecum could probably delay the digestion of the spermatophore, allowing more sperm to escape from the spermatophore tail into the upper spermoviduct, as has been suggested by Koene & Chase (1998a; 1998b) for the dart-shooting phenomenon of Helicoidea.

It has been suggested (Charnov, 1979) that calcium donation during copulation to the mating partner could be a nuptial gift for the production of eggs of the recipient, which would also increase the reproductive success of the donor. This hypothesis is based on the observation that many land pulmonates inject a calcium carbonate dart into the partners body (Hunt, 1979; Tompa, 1980; Leonard, 1992). We think this is not the case in *O. atlanticus*. The spermatophore of this species is free from the spherules stored in the male distal organs, having only a small amount of calcium on it. This hypothesis of the nuptial gift of calcium has also been rejected by Koene and Chase (1998a) for the spermatophore transference and dart shooting of *Helix aspersa*.

Other reasons that could explain the presence of these calcium phosphate deposits in the male distal organs of the reproductive system are related to the copulatory process itself. These deposits could participate in sexual stimulation of the mating partner, as has been proposed by Atkinson and Atkinson (1987) for the penial crystals of *Anguispira*. This stimulatory function has also been proposed for the calcareous envelope of *Cochlicella* (Schileyko & Menkhorst, 1997). It has been established that the courtship of stylommatophorans is characterized by several kinds of injuries and pheromone injections (Tompa, 1984; Adamo & Chase, 1990; Gómez, 1991) that serve to synchronize or accelerate the reciprocal copulation of mating pairs. The presence of free spherules inside the narrow space that occurs between penis and epiphallus during *O. atlanticus* copulation, together with the observed scratches on the surfaces of both organs, support a mechanical stimulatory function for the spherules. These scratches are common in the studied specimens of *O. atlanticus* that were killed during the copulation, and we think they are not an artefact.

More studies are required in order to know the extent of these granular cells in the genital system of molluscs, as well as to elucidate the possible function of these calcium and phosphate salts in the hardening of the spermatophore, in the copulatory process, or in other aspects of molluscan reproduction.

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